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EFFECTS OF CIGARETTE SMOKE EXTRACT ON LYMPHOCYTE FUNCTION

BACKGROUND

The worldwide use of tobacco, especially cigarette smoking, is a public health concern because of the increased frequencies of cancer and serious respiratory infections among smokers. While a problem is obvious, biological mechanisms leading to disease are not completely understood. That tobacco use is associated not only with the development of cancer, but also with increased susceptibility to respiratory infections (1,2) intimates that smoke components are not only carcinogenic, but also inhibitory to immune defenses. Mechanisms critical to fending off viral infections and emerging malignancies may be inhibited by tobacco smoke. The effect of smoking on immune status has been studied somewhat, but unclear and seemingly contradictory results prevail. An *in vitro* investigation of immunosuppressive mechanisms, which may lead to the problems observed *in vivo*, will be proposed.

Studies of smokers, or animals exposed to cigarette smoke, include descriptive observations and functional analyses of T lymphocytes, the central cellular elements of protection against infectious disease, and of natural killer cells (NK), lymphoid cells that play surveillance and effector roles in defense against malignancy. It is certain that tobacco smoke alters these cells in some way, but discrepancies in the results cloud the issue. For example, it has been shown in mice that chronic inhalation of cigarette smoke reduces T lymphocyte function in lung-associated lymph nodes, but in contrast, antigen presenting cells function normally in those lymph nodes (4). Other studies indicate that NK activity is depressed in smokers (3).

Many investigations of the effect of cigarette smoke on lymphoid cells use peripheral blood mononuclear cells (PBMC) from smokers, or from animals experimentally exposed to cigarette smoke. It has been suggested, however, that the immune elements affected most are localized in the lungs and regions nearby (4), and since PBMC do not represent the pulmonary region, this may account for much of the confusion. Isolation of more relevant cells, by bronchoalveolar lavage for example, would be more appropriate for studying the effects of cigarette smoke on immune function.

PRESENT STATUS AND GOALS

A recent editorial comment in The Lancet bemoaned the complexity of cigarette smoke and of the immune system and emphasized the difficulty of interpreting the results from the numerous studies (5). It was suggested that a more holistic approach to immune function is required. But rather than increasing our understanding of the immune status of smokers, this may only compound the complexity.

In contrast, a more precise understanding of underlying mechanisms involved in the effect of tobacco smoke on individual cells would clarify and increase our ability to understand the holistic picture. This could be done *in vitro* by applying tobacco smoke components to isolated normal lymphocytes (from non-smokers) and by studying the mechanism of effects. With this approach, it is possible to dissect the direct effects of the smoke on the cells and clarify our understanding of those underlying cellular mechanisms.

In addition, it will provide a model for studies of these mechanisms on a molecular level.

Preliminary experiments have been performed in this laboratory which demonstrate a definite effect of cigarette smoke on murine lymphocytes. A crude extract of cigarette smoke (CSE) was prepared, filter sterilized, and frozen for use in this study. Spleen cells (SC) from inbred BALB/c mice were treated with various concentrations of CSE and tested in separate experiments for a proliferative response to a mitogenic stimulus and for NK cell function.

In a dose-dependent fashion, CSE-treatment inhibited the proliferation of SC stimulated with concanavalin-A (Con-A). Untreated Con-A-stimulated SC served as controls.

A dose-dependent inhibition of NK function was also observed when the CSE-treated SC effectors were incubated with radioactively labeled YAC-1 target cells in a chromium-release assay for cell-mediated cytotoxicity.

Goals of this research effort include clarification of the effects cigarette smoke components have on immune functions that are critical to protection against cancer and infectious respiratory disease. Specifically, it will be determined whether cigarette smoke inhibits or alters normal lymphocyte functions such as proliferation, antigen-specific and non-specific cytotoxicity, and immune regulation through lymphokine production. For the functions that prove to be affected by CSE, the identity of the particular smoke components responsible will be sought. The reversibility of the effect and the molecular mechanisms involved will be studied.

LITERATURE CITED

1) Finklea JF, Hasselblad V, Sandifer SH, Hammer DI, Lowrimore GR. 1971. Cigarette smoking and acute non-influenzal respiratory disease in military cadets. Am J Epidemiol 93:457-462.
2) Anderson P. Pederson OF, Bach B, Bonde GJ. 1990. Serum antibodies and immunoglobulins in smokers and nonsmokers. Clin Exp Immunol 47:467-473.
3) Johnson JD, Houchens DP, Kluwe WM, Craig DK, Fisher GL. 1990. Effects of mainstream and environmental tobacco smoke on the immune system in animals and humans: A review. Crit Rev Toxicol 20(5):369-395.
4) Chang JCC, Distler SG, Kaplan AM. 1990. Tobacco smoke suppresses T cells but not antigen-presenting cells in the lung-associated lymph nodes. Toxicol Appl Pharmacol 102:514-523.
5) Smoking & immunity. 1990. Lancet 335(8705):1561-1563.

PROPOSED RESEARCH & ITS IMPACT

CSE will be prepared by bubbling mainstream smoke, from a burning cigarette, through liquid cell culture medium. The CSE will be filter sterilized and frozen in aliquots for later use. SC from inbred mice will either be treated by incubating them with serially diluted CSE or be sham-treated for controls. After washing the cells, the effect of CSE on various immune capabilities, as described below, will be tested *in vitro*:

- Proliferative response. Activated lymphocytes normally proliferate as part of immune responses. To confirm preliminary results already observed, CSE-treated and untreated SC will be stimulated with the plant

lectin concanavalin-A. At the appropriate time ^{3}H-thymidine will be added and incubation will continue. The cells will eventually be harvested and thymidine uptake will be determined with a liquid scintillation counter.

- Antigen-specific cytotoxicity. A key mechanism for fending off many viral infections and tumors is antigen specific cytotoxic T lymphocyte (CTL) function. TNP-specific cytotoxicity, which can be generated entirely *in vitro*, will be used to test the effect of CSE on this function. CTL precursors (normal SC) will be treated with CSE before an *in vitro* stimulation with irradiated TNP-modified SC in order to test the effect on the generation of CTL activity. In other experiments, CTL effectors generated with no prior CSE-treatment will instead be treated with CSE after the *in vitro* generation in order to test for the effect of the CSE at the effector level. A ^{51}Cr-release assay using TNP-modified SC as target cells will be used to measure cytotoxicity.
- NK activity. NK cells provide another cytotoxic effector mechanism important for protecting against viruses and cancers. Although not antigen-specific like CTL, NK cells preferentially kill virally-infected cells and tumor cells in a somewhat spontaneous fashion. To confirm preliminary results, NK activity will be induced in mice by inoculating them with a non-lethal dose of murine cytomegalovirus (MCMV). After 2 days, SC from these mice will be treated with the CSE and used as effectors in a ^{51}Cr-release assay with YAC-1 cells as targets. Untreated effector cells will be used as controls.
- Lymphokine production. Regulation of the complex immune response is partially orchestrated by soluble factors produced by lymphocytes. When CSE-treated SC are stimulated with concanavalin-A, supernatants will be collected from the cultures and analyzed for several key lymphokines, for example IL-2, IL-4, TNF-alpha, and IFN-gamma. The production of lymphokines by CSE-treated cells will be quantified using monoclonal antibodies in enzyme-linked immunosorbent assays (ELISA).

When CSE is found to alter or inhibit a normal lymphocyte function, other questions will be asked. The reversibility of CSE's effect will be tested by extending the incubation of treated SC, after washing them, as long as possible before repeating the functional assay. Also, an effort will be made to identify specific smoke components or derivatives (e.g. cotinine) which might be specifically responsible for the observed effect. Finally, when effects are seen, pertinent molecular studies will be conducted in an attempt to discover changes in proteins or gene expression.

This study will be free from unknown *in vivo* influences, so that the "potential" *in vivo* effect will be clearly understood, and will also be easily exploited for investigation of molecular mechanisms.

What might the impact of information obtained by these experiments be? In the long run, a clearer understanding of how tobacco smoke affects the immune system will lead to more effective treatment of patients and better public education regarding the effects of smoking.

ESTIMATED DURATION & DIRECT COSTS

It is anticipated that the work proposed for this project will take 3 years to complete with an estimated direct cost of about $25,000 per year.

CURRICULUM VITAE & BIBLIOGRAPHY

Roger L. Noble, Ph.D., Assistant Professor of Medical Education & Biology

Degrees & Training

Brigham Young University, Provo, Utah	B.S. 1978	Microbiology
Utah State University, Logan	M.S. 1983	Virology
Utah State University	Ph.D. 1986	Immunology
University of Michigan, Ann Arbor	Post-doc	85-87 Transplant Immun
Utah State University	Post-doc	87-88 Viral Immunology

Research and Professional Experience:

Department of Biology, Utah State University, Logan - Graduate Research/Teaching Assistant, 1978-1985; teaching microbiology & immunology labs; rotavirus & nutrition; Down's syndrome & immunity.

Transplantation Society of Michigan, Ann Arbor - Research Fellow, 1985; cytotoxic T cell-mediated cutaneous lesions.

Department of Surgery, University of Michigan Medical School, Ann Arbor - Research Fellow, 1985-1987; cytotoxic T cell-mediated cutaneous lesions.

Department of Animal, Dairy & Veterinary Sciences, Utah State University - Rsch Associate, 1987-88; immunity to arenaviruses & cytomegaloviruses.

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis - Adjunct Assistant Professor, 1988-present.

Muncie Center for Medical Education, Ball State University, Muncie, Indiana - Assistant Professor, 1988-present; cytomegaloviruses; tobacco smoke & immunity; - Chairman, Institutional Biosafety Committee, 1989-present

Honors:

- Student Research Paper Award, 1980 Meeting - ASM Intermountain Branch
- Travel Award, 8th Int'l Congress of Immunology, Budapest - AAI

Publications:

1. **Noble RL, Sidwell RW, Mahoney AW, Barnett BB and Spendlove RS.** Influence of malnutrition and alterations in dietary protein on murine rotaviral disease. Proc Soc Exp Biol Med 173:417-426. 1983.
2. **Noble RL.** Murine rotavirus infection: Characterization of the disease and influence of host malnutrition and dietary protein. M.S. Thesis. Utah State University. 95 pp. 1983.
3. **Morrey JD, Sidwell RW, Noble RL, Barnett BB and Mahoney AW.** Effects of folic acid malnutrition on rotaviral infection in mice. Proc Soc Exp Biol Med 176:77-83. 1984.
4. **Noble RL and Warren RP.** Age-related development of human natural killer cell activity. N Engl J Med 313:641-642. 1985.
5. **Noble RL.** Cellular immunity in children with Down syndrome (trisomy-21). Ph.D. Dissertation. Utah State University. 83 pp. 1985.

6. **Noble RL and Warren RP.** Altered T-cell subsets and defective T-cell functions in young children with Down syndrome (trisomy-21). Immunological Investigations 16(5):371-382. 1988.
7. **Noble RL and Warren RP.** Analysis of blood cell populations, plasma zinc and natural killer activity in young children with Down syndrome. J Ment Defic Res 32(June):193-201. 1988.
8. **Steinmuller D, Tyler JD, Snider ME, Noble RL, Riser BL, Maassab HF and Galli SJ.** Tissue destruction resulting from interaction of cytotoxic T cells and their targets. Ann New York Acad Sci 523:106-118. 1988.
9. **Noble RL and Steinmuller D.** Blocking of interleukin-2 production, but not the tissue destruction induced by cytotoxic T cells, by cyclosporine. Transplantation 47:322-326. 1989.
10. **Steinmuller D, Snider ME, Noble RL and Waldschmidt TJ.** Dissociation of tissue destruction induced by cytolytic T cells in vivo and cytotoxicity as measured in vitro. Transplantation 50:663-668. 1990.
11. **Steinmuller D, Snider ME, Noble RL, Tyler JD and Galli SJ.** The murine immune lymphocyte transfer reaction revisited. Transplantation Proceedings 23(1):163-164. 1991.
12. **Smee DF, Pease A, Burger RA, Huffman JH, Morrey JD, Okleberry KM, Noble RL and Sidwell RW.** Ganciclovir treatment of murine cytomegalovirus infection in mice immunosuppressed by prior infection with Friend leukemia virus. Antiviral Chemistry & Chemotherapy 3(6):327-333. 1992.
13. **Noble RL & Stone ME.** Abrogation of Lethal Murine Cytomegalovirus Infection by Passive Transfer of Cytolytic Effector Cells. Immunology and Cell Biology (submitted for publication Jan 1993).